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Molecular Cloning and Tissue-specific Expression of Cu/Zn and Mn-superoxide Dismutase in the Three-keeled Pond Turtle, *Chinemys reevesii*

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Abstract Both copper/zinc superoxide dismutase (SOD; Cu/Zn-SOD, SOD1) cDNA and manganese SOD (Mn-SOD, SOD2) cDNA were cloned for the first time from the three-keeled pond turtle, *Chinemys reevesii,* using RT-PCR and RACE methods in this work. The SOD1 cDNA was 749 bp long and consisted of a 32-bp 5'-untranslated region (UTR), a 249-bp 3'-UTR, and a 468-bp open reading frame (ORF) encoding a 155-amino-acid protein with 16.0 kDa predicted molecular mass and 5.95 theoretical isoelectric point (p*I*). The SOD2 cDNA was 1687 bp long and comprised 94-bp of 5'-UTR, 912-bp 3'-UTR and 681-bp ORF encoding a 226-amino-acid protein with 25.0 kDa predicted molecular mass and 8.83 p*I*. The deduced amino acid sequence of SOD1 showed relatively high similarity (77.4%–87.1%) and identity (65.4%–74.4%) with the published sequences of SOD1 from other vertebrate species, whereas SOD2 protein shared slightly higher similarity (83.6%–95.6%) and identity (76.1%–88.9%) with other reported vertebrates SOD2s. Phylogenetic analysis revealed that the *C. reevesii* SOD1 and SOD2 were separately clustered together, and were highly conserved during evolution. Both SOD mRNA expression was detected widely in the brain, liver, muscle, kidney, gut, spleen, lung and heart at variable levels. The highest expression of the two SODs was observed in muscle, and followed in brain, liver, kidney, gut and heart, whereas low transcriptional levels were found in spleen and lung. Meanwhile, high activity of SOD1 was kept in brain, liver, muscle, kidney and heart, and followed in gut, spleen and lung. The activities of SOD2 in brain, liver, muscle, kidney, gut and heart were significantly higher than those in spleen and lung.

Keywords *Chinemys reevesii*, SOD1, SOD2, cDNA cloning, mRNA expression

1. Introduction

Generally, reactive oxygen species (ROS) are constantly generated in organisms as side-products of oxygen metabolism (Winterbourn, 2008). ROS such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are oxygen-derived molecules with strong oxidative properties (Bandyopadhyay *et al.*, 1999). Under some circumstances, the dynamic balance between ROS production and elimination is disturbed, leading to overproduction of ROS called oxidative stress (Lushchak, 2011a). The excessive stimulation of ROS causes harmful damage to cellular macromolecules, including lipids, proteins, carbohydrates and nucleic acids, which result in cell death and tissue physiological damage (Valko *et al.*, 2007). To overcome oxidative damage, aerobic organisms develop highly powerful antioxidant defense system against ROS to maintain redox homeostasis.

Superoxide dismutase (SOD; EC 1.15.1.1) is considered as the first and most important defense line against oxidative stress by catalyzing dismutation of O_2^- into dioxygen and H_2O_2, which is subsequently converted into H_2O by the action of catalase and glutathione peroxide (Afonso *et al.*, 2007). Three distinct types of SODs have been characterized based on the metal cofactor at the catalytic sites: copper/zinc (Cu/Zn-SOD; SOD1), manganese (Mn-SOD; SOD2), and iron (Fe-SOD) (Fridovich, 1986). Two kinds of SODs have been

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found inside eukaryotic cells, with SOD1 generally existing in the cytoplasm and outer mitochondrial space, and SOD2 exclusively in the inner mitochondrial space (Landis and Tower, 2005). So far, successful genetic characterization have been completed in several fishes, amphibians, birds and mammals (Landis and Tower, 2005). However, no complete SOD cDNA sequences have been cloned in reptiles yet. Thus, more information at the genetic level would be helpful to obtain better insights into the mechanisms of SOD gene regulation.

It has been proven that strong environmental stress is usually accompanied by oxidative stress, which increases the risk of tissue injury, then followed by physiological consequences like diseases, and finally death in aquatic animals (Lushchak, 2011b). SODs with the ability to catalyze the dismutation of the toxic superoxide anion play a key role in antioxidant defense systems (Fridovich, 1995). Since SODs were first described as enzymes for erythrocuprein in 1969 (McCord and Fridovich, 1969), they have now been well investigated for each physiological function and roles of them in protection against ROS (Fridovich, 1986, 1995; Afonso *et al.*, 2007). The expression and activity of SOD have also been characterized to be suitable oxidative stress indicators for assessing the environmental risk in many aquatic animals (Almeida *et al.*, 2002; Pandey *et al.*, 2003; Valdivia *et al.*, 2007; Zhang *et al.*, 2011).

The Three-keeled pond turtle, *Chinemys reevesii* is a prime reptile species for studying the antioxidant response to the aquatic environment. It is a widely distributed reptile with great economic value in China, and therefore it is commercially cultured. In the intensive aquaculture, turtles often undergo environmental stressors including high stocking density, temperature change and ammonia contamination. Despite numerous studies focusing on the adaptive change of SOD activity in aquatic animals exposed to environmental stress, only a few researchers have combined their results with the analysis of gene expression at the mRNA level (Hansen *et al.*, 2006; Lushchak and Bagnyukova, 2006; Craig *et al.*, 2007).

In this study, Cu/Zn-SOD and Mn-SOD full-length cDNA of *C. reevesii* were cloned and molecularly characterized. The tissue-specific mRNA expression and enzyme activities of adults were also analyzed. This is the first report on the sequences of SOD genes, and their tissue-specific expression pattern in turtle.

2. Materials and Methods

2.1 Turtle sample Healthy *C. reevesii* adults (mean weight (348.46 ± 57.33 g) were purchased from the Shilihe Pet Market (Beijing, China). They were reared and maintained in tanks at 28 °C ± 1 °C with 13 h/11 h light/darkness cycles. The standard diet (Haitai Technology Limited Company, Shijiazhuang, Hebei, China) was supplied to feed the turtles once a day for 4 weeks. Uneaten food was carefully removed 1 h after feeding, and 50% tank water was replaced every day. Tissues (brain, liver, muscle, kidney, gut, spleen, lung and heart) from eight adult turtles were sampled individually under the inspection of the Animal Welfare Council of Beijing Normal University on 14 May 2012. Freshly dissected tissues were frozen in liquid nitrogen immediately and stored at -80 °C until used.

2.2 Total RNA extraction and cDNA synthesis Total RNA was isolated from the tissues stated above and genomic DNA was removed using NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany). RNA concentration and purity were measured by NanoVue™ ultraviolet spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Meanwhile, RNA integrity was ensured by analysis on a 1.0% agarose gel. According to the manufacturer's instructions, 3'-Ready cDNA and 5'-Ready cDNA syntheses were respectively performed using SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). And single-strand cDNA for SOD1 5'-RACE was also synthesized from 5.5 μg total RNA using a SOD1 5'-RACE RT primer (Table 1). All primers used in this study are shown in Table 1.

2.3 Full-length cDNA cloning of *C. reevesii* SODs genes Degenerate primers (SOD1 F1/R1 and SOD2 F1/R1) based on the conserved domains of the two genes from other reported vertebrates were designed (Table 1). PCR was performed by denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 1.5 min, and an additional elongation at 72 °C for 7 min. The target RT-PCR products were purified from 1.0% agarose gel using the SanPrep DNA gel extraction kit (Sangon, Shanghai, China), cloned into *pEASY*-Simple vector (Transgen, Beijing, China), and transformed into *Trans5α* chemically competent cells (Transgen, Beijing, China). Sequences from five independent clones were verified for the correct insert.

Based on the sequences from a conserved region we got in *C. reevesii*, 3'- and 5'-RACE primers (Table 1) were designed. 3'-RACE for SODs was performed with primers SOD1 32 and SOD2 32, and then followed by nested PCR with SOD1 33 and SOD2 35, respectively. Likewise,

primer SOD2 52 was used for SOD2 5'- RACE, as well as SOD2 51 for SOD2 nested PCR. The amplification of the 5'-end of SOD1 cDNA was performed in two parts. Primer SOD1 55 and 5' Ready cDNA were used for SOD1 5'- RACE, and then followed by nested PCR with SOD1 53. At the same time, SOD1 5'-RACE was also amplified using primer SOD1 58 and SOD1 5' Ready cDNA, followed by nested PCR using primer SOD1 57. A series of RACE and nested PCR was conducted with the SMART™ RACE cDNA Amplification Kit and Phusion® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA, USA). Touchdown PCR was adopted to improve the specificity of SMARTer RACE amplification. A series of RACE PCR were performed with 1 cycle of 1 min at 98 °C, 11 cycles of (10 sec at 98 °C; 30 sec at 72 °C, -1 °C each cycle; 1 min at 72 °C), followed by 24 cycles of (10 sec at 98 °C; 30 sec at 61 °C; 1 min at 72 °C), and 5 min at 72 °C after the last cycle. The conditions for the nested PCR were 98 °C for 1 min, 12 cycles of (98 °C for 10 sec; 72 °C for 30 sec, -1.5°C / 2 cycles; 72°C 1 for min), followed by 23 cycles of (98 °C for 10 sec; 63 °C for 30 sec; 72 °C for 1 min) and 72 °C for 5 min after the last cycle. Finally, two pairs of gene-specific primers, namely, SOD1ORF F/R and SOD2ORF F/R (Table 1), were synthesized to amplify the complete coding sequence (CDS). Parameters used for verification PCR were the same as mentioned above. Both the RACE and verification PCR products were excised from 1.0% agarose, cloned into *pEASY*-Blunt vector (Transgen, Beijing, China) and sequenced.

2.4 Sequence characterization and phylogenetic analysis The whole sequences of two turtle SODs were further analyzed with the online BLAST program from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein analyses were performed using ExPASy online tools (http://us.expasy.org/tools). MITOPROT (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) was used to find SOD2 mitochondria targeting sequence (MTS). Multiple alignments of SOD protein sequences were carried out using ClustalX2 (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) and edited using BioEdit 7.0.8 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Similarity and identity at amino acid level were calculated using the Matrix Global Alignment Tool (MatGAT v2.03, http://bitincka.com/ledion/matgat/). A phylogenetic tree was constructed by the neighbor-joining (N-J) method in Mega 5.0 with 1000 bootstraps (Tamura *et al.*, 2011). Species and accession numbers for sequence alignment and phylogenetic analysis are shown in Table 2.

2.5 Tissue expression analysis The tissue distributions of SOD1 and SOD2 mRNA were studied by using semi-

Table 1 Primers used for *C. reevesii* SOD1 and SOD2 gene cloning and RT-PCR analysis.

Gene	Primer	Sequence (5'-3')	Application
SOD1	SOD1 5'-RACE RT primer	TCCATTATCCTGCTGCTC	SOD1 5' Ready cDNA synthesis
	SOD1 F1	CSGTGTGYGTGMTGAAG	Cloning
	SOD1 R1	ARRCGAGSWCCAGCATTKCC	
	SOD1 32	ACGCTCTCAGGAAGGATTACAGGTTTG	3'-RACE
	SOD1 33	GCAGGTGCTCACTTCAATCCTAATGGC	
	SOD1 55	CCAGACGACCACCAGCATTGCCAGTT	5'-RACE
	SOD1 53	GGTGCGTCCAATAACAGAAAGCGGTC	
	SOD1 58	TCCATTATCCTGCTGCTCGAAGTTG	
	SOD1 57	TCTCTCCCTTCAGCACACACACCG	
	SOD1ORF F	ACATGGCGGCGGTGAAGGC	CDS verification
	SOD1ORF R	CCAGAACAAGGCCAAAACATTGATGT	
	SOD1 F	CGCTCTCAGGAAGGATTACAG	RT-PCR
	SOD1 R	AATAACACCACAAGCCAGACG	
SOD2	SOD2 F1	ARAARCACACTCTTCCTGAC	Cloning
	SOD2 R1	CCTTGAACACCAACYGATAC	
	SOD2 32	AAGAGGCATTGGCAAAAGGTGATGTTAC	3'-RACE
	SOD2 35	GCAAAAGGTGATGTTACAGCTCAGGTG	
	SOD2 52	TTGTCCAGAAGATGGTGTGGTTGATGTG	5'-RACE
	SOD2 51	CTGTAACATCACCTTTTGCCAATGCCTC	
	SOD2ORF F	GGAGCCCGAGCCACCGCCAT	CDS verification
	SOD2ORF R	GAGTAGAACTGGTGCTCTTGTAATAGTCTGTGTG	
	SOD2 F	ACACACTCTTCCTGACTTGCC	RT-PCR
	SOD2 R	CCATCCTGAGCCTTGAACAC	
Actin	Actin F	TTGTCCCTGTATGCCTCTGGT	RT-PCR
	Actin R	TGGTTTCATGAATGCCACAGG	

quantitative RT-PCR. Total RNA from brain, liver, muscle, kidney, gut, spleen, lung and heart was extracted as described above. First-strand cDNA was synthesized using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa BIO, Otsu, Shiga, Japan). The total amount of cDNA was calibrated on the basis of amplification of *C. reevesii* β-actin. Pairs of specific primers were SOD1 F/R, SOD2 F/R and Actin F/R, respectively, to amplify either 358 bp of SOD1, 366 bp of SOD2 or 377 bp of β-actin gene segments (Table 1). The reaction was cycled under the same conditions of sequence cloning. The products of the PCR were visualized by electrophoresis of SYBR Green I stained 1.0% agarose gel.

2.6 Measurement of SOD activities The total SOD and SOD1 activities in brain, liver, muscle, kidney, gut, spleen, lung and heart were assessed with SOD detection

Table 2 Similarity and identity of *C. reevesii* two genes with homologs of other reported vertebrates at amino acid level. The highest and the lowest are highlighted in bold. Matrix Global Alignment Tool (MatGAT v2.02) is applied in the calculation. Accession numbers are supplied.

	Common name	GenBank No.	Similarity (%)	Identity (%)
Species for SOD1 analysis				
Hypophthalmichthys molitrix	Silver carp	ADJ67808.1	82.6	71.8
Xiphophorus hellerii	Green swordtail	ADJ57704.1	80	66.7
Hypophthalmichthys nobilis	Big head carp	ADJ67809.1	81.9	71.2
Rachycentron canadum	Cobia	ABI96913.1	80.6	67.9
Epinephelus malabaricus	Malabar grouper	AAK62563.1	79.4	**65.4**
Hemibarbus mylodon	Korean doty barbel	ACR56338.1	81.9	70.5
Ctenopharyngodon idella	Grass carp	ADF31307.1	83.2	72.4
Oncorhynchus mykiss	Rainbow trout	NP_001117801.1	81.3	66.7
Salmo salar	Atlantic salmon	NP_001117059.1	79.4	67.3
Danio rerio	Zebrafish	NP_571369.1	79.4	67.3
Xenopus (Silurana) tropicalis	Western clawed frog	NP_001016252.1	79.4	70.3
Melopsittacus undulatus	Budgerigar	AAO72711.1	84.5	72.9
Gallus gallus	Chicken	NP_990395.1	85.2	72.3
Mus musculus	House mouse	NP_035564.1	86.5	**74.4**
Rattus norvegicus	Norway rat	NP_058746.1	**87.1**	73.7
Oryctolagus cuniculus	Rabbit	NP_001076096.1	80	71.6
Canis lupus familiaris	Dog	NP_001003035.1	81.9	70.3
Bos taurus	Cattle	NP_777040.1	80	71.6
Ovis aries	Sheep	NP_001138657.1	79.4	71
Sus scrofa	Pig	NP_001177351.1	**77.4**	67.1
Equus caballus	Horse	NP_001075295.1	83.2	72.4
Macaca mulatta	Rhesus monkey	NP_001027976.1	85.2	73.7
Pan troglodytes	Chimpanzee	NP_001009025.1	83.9	72.4
Pongo abelii	Sumatran orangutan	NP_001125441.1	83.9	72
Homo sapiens	Human	NP_000445.1	83.9	72.4
Species for SOD2 analysis				
Danio rerio	Zebrafish	NP_956270.1	86.7	78.3
Paralichthys olivaceus	Japanese flounder	BAJ79013.1	86.3	76.2
Rachycentron canadum	Cobia	ABC71306.2	86.7	76.7
Xenopus (Silurana) tropicalis	Western clawed frog	NP_001005694.1	89.4	81.9
Xenopus laevis	African clawed frog	NP_001083968.1	88.1	81.4
Melopsittacus undulatus	Budgerigar	AAO72712.1	95.6	88.9
Gallus gallus	Chicken	NP_989542.1	94.2	88.1
Mus musculus	House mouse	NP_038699.2	89.4	82.4
Rattus norvegicus	Norway rat	NP_058747.1	89.4	82.4
Cavia porcellus	Domestic guinea pig	XP_003466415.1	90.3	82.7
Canis lupus familiaris	Dog	XP_533463.3	89.4	81.4
Bos taurus	Cattle	NP_963285.2	90.7	83.6
Ovis aries	Sheep	ACV04835.1	92	84.1
Sus scrofa	Pig	NP_999292.2	90.7	84.5
Equus caballus	Horse	NP_001075986.1	90.7	82.7
Macaca mulatta	Rhesus monkey	NP_001027977.1	84.1	76.1
Pan troglodytes	Chimpanzee	NP_001009022.1	83.6	76.1
Pongo abelii	Sumatran orangutan	NP_001127035.1	89.8	82.3
Homo sapiens	Human	AAH12423.1	89.8	82.3

kit (Jiancheng Co., Nanjing, Jiangsu, China). One unit of T-SOD or SOD1 activity was defined as the amount of protein required to inhibit the rate of reduction of SOD by 50% under the assay conditions. The enzyme activities were expressed as U/mg of protein (U/mg protein). SOD2 activity was calculated as the difference between T-SOD and SOD1 activities. Total proteins were determined using Coomassie brilliant blue method with bovine serum albumin as a reference protein (Jiancheng Co., Nanjing, Jiangsu, China).

2.7 Statistical analysis The data of SODs activities were expressed as mean ± S.E. Statistical differences were determined by one-way ANOVA followed by the LSD test. Significance was accepted at $P < 0.05$. The SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) software package was used for all analysis.

3. Results

3.1 Cloning and characterization of SOD1 and SOD2 cDNA The full-length cDNA of *C. reevesii* SOD1 and SOD2 genes were completely sequenced and had been submitted to GenBank (Accession Nos. JX843790 for SOD1 and JX843791 for SOD2).

The complete cDNA of SOD1 was 749 bp, including 32 bp of 5'-untranslated region (UTR), 468 bp of open reading frame (ORF), and 249 bp of 3'-UTR (Figure 1 A). The ORF encoded a 155-amino-acid protein with predicted molecular weight and theoretical isoelectric point (p*I*) in 16.0 kDa and 5.95, respectively. The 3'-UTR contained a polyadenylation signal, ATTAAA. Two SOD1 family conserved signature sequences were found: ^{45}GFHVHEFGDNT^{55} and ^{139}GNAGGRLACGVI^{150} (Figure 1 A). Additionally, *C. reevesii* SOD1 amino acid sequence contained putative Cu ligands (His-47, -49, -64, and -121), Zn ligands (His-64, -72, -81, and Asp 84) and two Cys residues (Cys-58 and -147) forming a disulfide bridge, which were typical characteristics of all other SOD1.

The SOD2 cDNA sequence was cloned as 1687 bp, containing a 94-bp 5'-UTR, a 912-bp 3'-UTR, and 681-bp ORF encoding a 226-amino-acid-residue protein with calculated Mw 25.0 kDa and a theoretical p*I* of 8.83. No polyadenylation signal was found before the poly (A) tail, whereas signature sequence was observed from 187 to 194 residues (DVWEHAYY). Conserved domains required for metal binding were also detected in *C. reevesii* SOD2 (His-54, -102, -191, and Asp-187). In addition, the predicted mitochondria targeting sequence (MTS) (MALLCRLASRGRS) was located at the N'-terminal region (Figure 1 B).

3.2 Comparison and phylogenetic analysis Multiple sequence alignments showed a high degree of sequence conservation among *C. reevesii* SOD1 and SOD2 and those of other vertebrate species, respectively (Figure 2 A, B). The amino acid residues coordinating metal ions were highly conserved across species (Figure 2 A, B). As shown in Table 2 A, the *C. reevesii* SOD1 protein shared relatively high similarity (77.4%–87.1%) and identity (65.4%–74.4%) compared with others. SOD1 from *C. reevesii* showed higher similarity and identity when compared with SOD1 from the Norway rat *Rattus norvegicus* (87.1%, 73.7%), house mouse *Mus musculus* (86.5%, 74.4%), rhesus monkey *Macaca mulatta* (85.2%, 73.7%), budgerigar *Melopsittacus undulatus* (84.5%, 72.9%) and chicken *Gallus gallus* (85.2%, 72.3%), respectively. SOD2 protein shared slightly higher similarity (83.6%–95.6%) and identity (76.1%–88.9%) with other SOD2s (Table 2 B). The deduced amino acid sequence of *C. reevesii* SOD2 had higher similarity and identity with *M. undulatus* (95.6%, 88.9%), *G. gallus* (94.2%, 88.1%), domestic guinea pig *Cavia porcellus* (90.3%, 82.7%), cattle *Bos taurus* (90.7%, 83.6%), sheep *Ovis aries* (92%, 84.1%), pig *Sus scrofa* (90.7%, 84.5%) and horse *Equus caballus* (90.7%, 82.7%), respectively.

Phylogenetic reconstruction showed supports that the turtle's SOD1 and SOD2 were separately evolved and corresponded with conventional taxonomy (Figure 3). The *C. reevesii* SOD1 protein located in the SOD1 cluster formed a sub-cluster with the western clawed frog, *Xenopus (Silurana) tropicalis*, while SOD2 clustered with a large vertebrate sub-cluster, to the exclusion of fishes.

3.3 Tissue distribution patterns of SOD1 and SOD2 Both SODs were detected in all eight tissues examined (brain, liver, muscle, kidney, gut, spleen, lung and heart). The highest expression for both SODs was observed in muscle, and moderate expression levels were found in brain, liver, kidney, gut, and heart, whereas low transcriptional levels were present in the spleen and lung.

3.4 SOD activity assays Significant differences of SOD1 activity was found among distinct tissues (Figure 5 A; $F = 19.382$, $df = 63$, $P < 0.001$). SOD1 activity in the brain ($P = 0.002$, $P < 0.001$, $P < 0.001$), liver ($P < 0.001$, $P < 0.001$, $P < 0.001$), muscle ($P < 0.001$, $P < 0.001$, $P < 0.001$), kidney ($P < 0.001$, $P < 0.001$, $P < 0.001$) and heart ($P = 0.001$, $P < 0.001$, $P < 0.001$) were higher than those in gut, spleen and lung, respectively. Meanwhile, SOD1 activity in the spleen ($P = 0.003$) and lung ($P = 0.005$) were significantly lower than those in the gut.

Figure 1 The nucleotide and deduced amino acid sequences of *C. reevesii* SOD1 (A) and SOD2 (B). The stop codon is indicated by an asterisk (*) and the SOD1 polyA signal (ATTAAA) in the 3'-UTR is shown by a box. SOD1 family conserved signature sequences (-GFHVHEFGDNT- and -GNAGGRLACGVI-) and SOD2 family conserved signature sequence (-DVWEHAYY-) are underlined. The MTS sequence of SOD2 appears as double-lined.

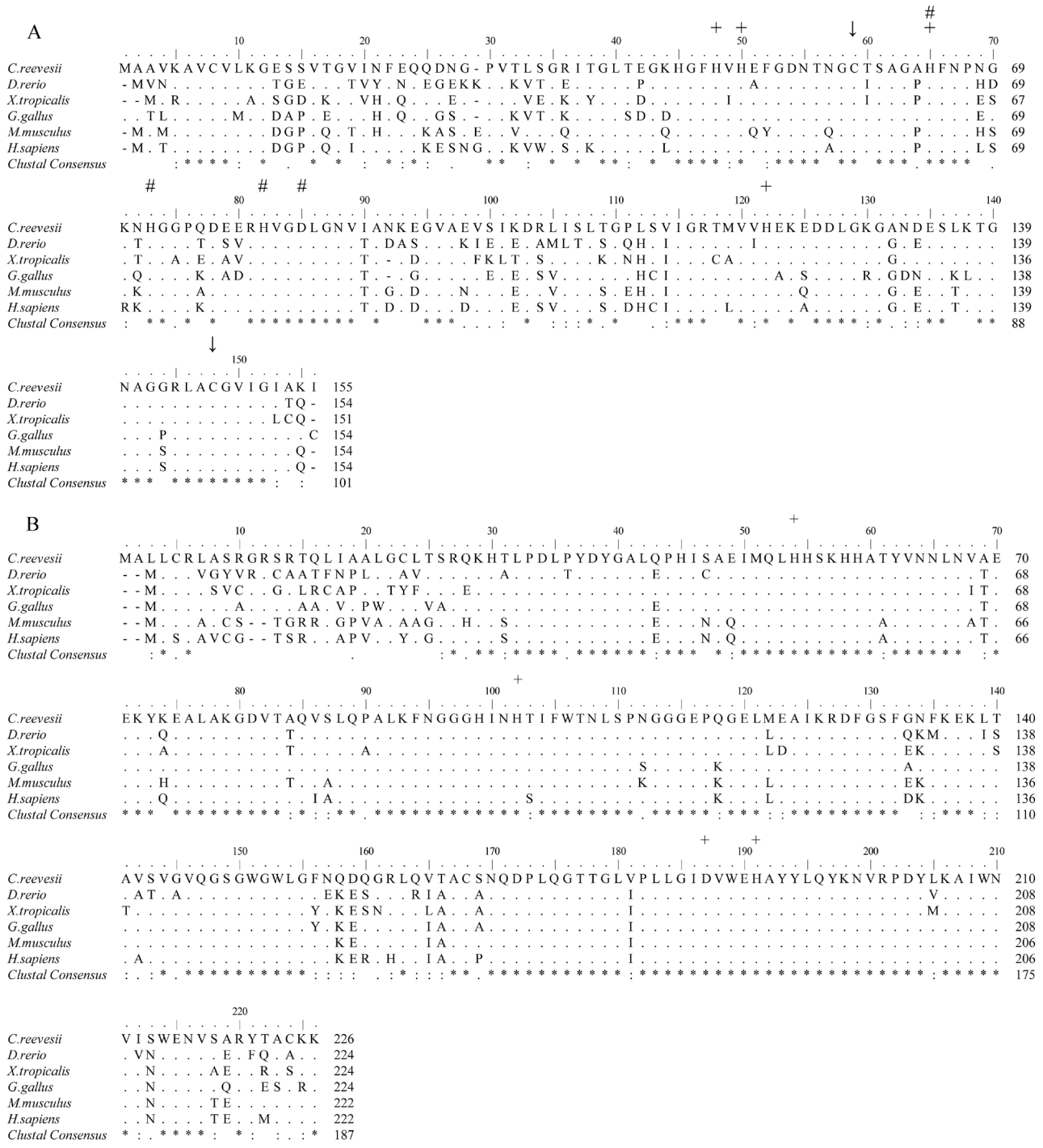

Figure 2 Multiple sequence alignment of SOD1 (A) and SOD2 (B) between *C. reevesii* and selected vertebrates at amino acid level. The consensus residues are shaded with a threshold of 50% identity. In the consensus line, asterisks (*) represent completely identical residues in all selected vertebrates, and dots (.), and colons (:) resemble similarity as described in the general information of the CluxtalX2 program. A: + denotes amino acids responsible for binding copper, # for zinc, two cysteines forming a disulfide bridge are indicated with arrows; B: + refers to manganese-binding residues. Accession numbers are supplied in Table 2.

However, no differences were observed among enzyme activity in the brain ($P > 0.05$), liver ($P > 0.05$), muscle ($P > 0.05$), kidney ($P > 0.05$) and heart ($P > 0.05$), nor between spleen and lung ($P > 0.05$).

SOD2 activity also differed significantly among tissues (Figure 5 B; $F = 6.325$, $df = 63$, $P < 0.001$). SOD2 activity in the brain ($P < 0.001$, $P < 0.001$), liver ($P < 0.001$, $P < 0.001$), muscle ($P = 0.001$, $P < 0.001$), kidney ($P < 0.001$, $P < 0.001$), gut ($P = 0.006$, $P = 0.003$) and heart ($P = 0.001$, $P < 0.001$) were significantly higher

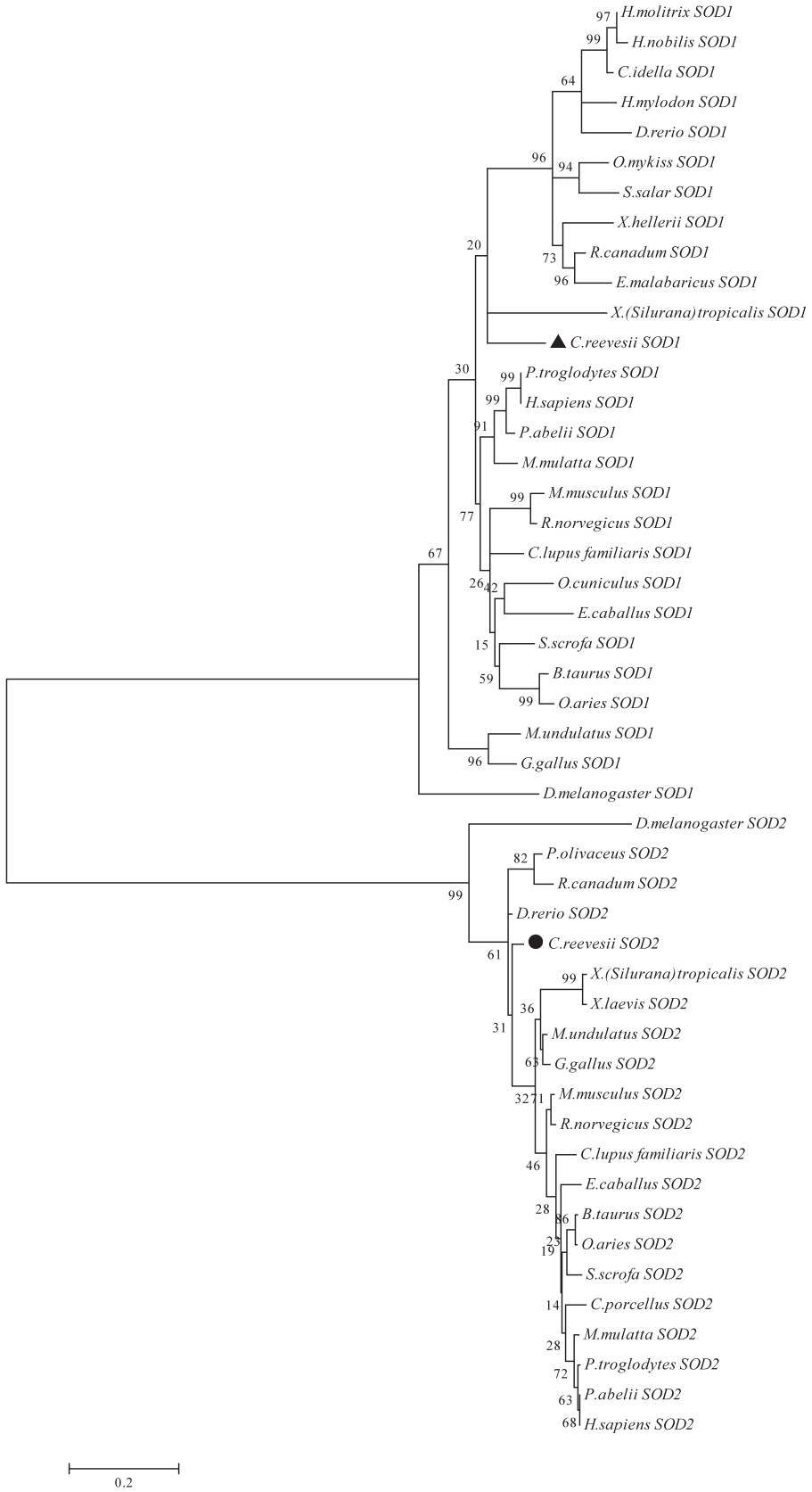

Figure 3 Phylogenetic relationships of SOD1 and SOD2. The phylogram was constructed by ClustalX2 and Mega 5.0 using the neighbor-joining method with bootstrap values of 1000. The accession numbers of the fruit fly, *Drosophila melanogaster* are: SOD1 NP_476735.1; SOD2 NP_476925.1.

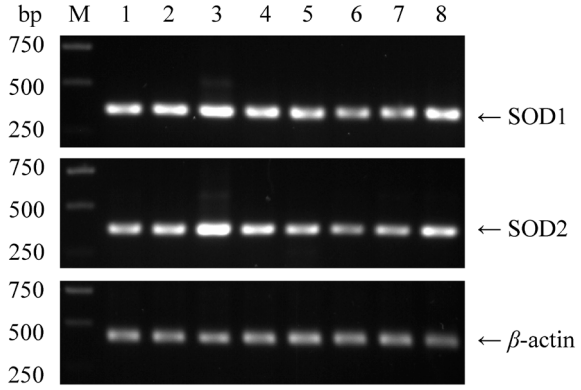

Figure 4 Tissue-specific expressions of SODs genes in eight tissues of *C. reevesii*. M: DL2000 marker; 1: brain; 2: liver; 3: muscle; 4: kidney; 5: gut; 6: spleen; 7: lung; and 8: heart.

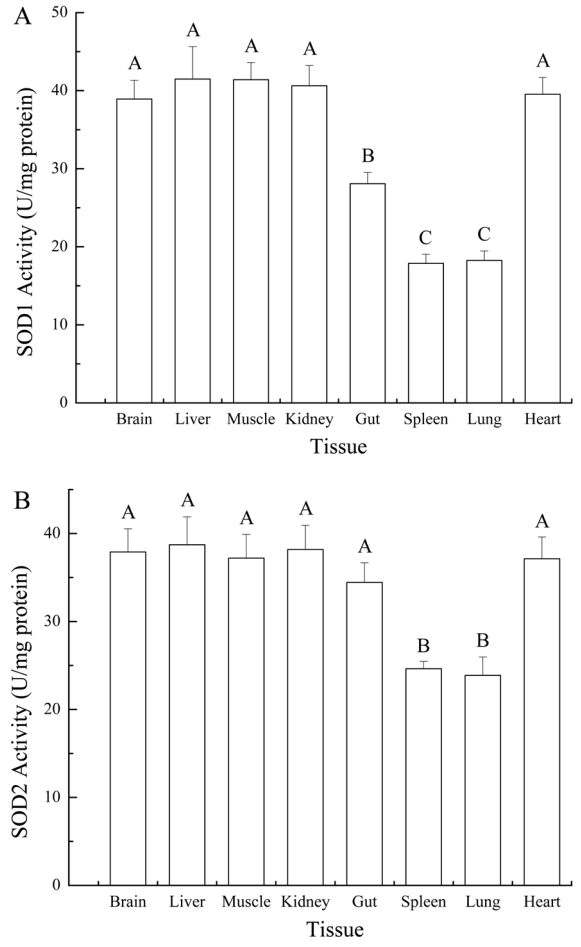

Figure 5 Specific activities of SODs in *C. reevesii* eight tissues (mean ± S.E.). A: SOD1; B: SOD2; n = 8. Different letters denote significant differences ($P < 0.01$).

than those in spleen and lung, respectively. No statistically significant differences were found among enzyme activity in the brain ($P > 0.05$), liver ($P > 0.05$), muscle ($P > 0.05$), kidney ($P > 0.05$), gut ($P > 0.05$), and heart ($P > 0.05$), nor between spleen and lung ($P > 0.05$).

4. Discussion

The obtained *C. reevesii* SOD1 protein sequence retains the conserved characteristics of previously reported SOD1 proteins (Yuan, 2009). The deduced amino acid sequence revealed four Cu^{2+} binding sites (His-47, -49, -64, and -121) and four Zn^{2+} binding sites (His-64, -72, -81, and Asp 84), as well as two Cys (Cys-58 and -147), which form a single disulfide bridge. Additionally, two SOD1 signature motifs were found in *C. reevesii* SOD1. Similar results were also observed in the zebrafish *Danio rerio* (Ken *et al.*, 1998), silver carp *Hypophthalmichthys molitrix* (Zhang *et al.*, 2011), river pufferfish *Takifugu obscurus* (Kim *et al.*, 2010), and a cartilaginous shark *Scyliorhinus torazame* (Nam *et al.*, 2006) SOD1s. Similarly, the deduced protein of *C. reevesii* SOD2 also had several key conserved features present in the SOD2 families (Abreu and Cabelli, 2010). Conserved manganese ion combining sites (His-54, -102, -191, and Asp-187) were also found along with a signature sequence and a MTS sequence. This is consistent with SOD2 described for *D. rerio* (Lin *et al.*, 2009), *H. molitrix* (Zhang *et al.*, 2011), *T. obscurus* (Kim *et al.*, 2010), and the Japanese flounder *Paralichthys olivaceus* (Wang *et al.*, 2011). Moreover, it was notable that two *C. reevesii* SODs shared considerably high similarity and identity with SODs from amphibians, birds and mammals, respectively. *C. reevesii* SOD1 was closely related to *X. (Silurana) tropicalis*, and SOD2 clustered with a large group of animals, including amphibians, birds and mammals. Particularly, the different evolutionary rates and the rapid sequence divergence of SOD1 were also partly revealed by the homology analysis and molecular evolutionary analysis (McCord and Fridovich, 1969; Kim *et al.*, 2010). Therefore, all these findings suggested that SOD1 and SOD2 were well conserved throughout evolution, especially in vertebrates.

In the present study, the *C. reevesii* SODs were observed with wide distribution patterns at both transcription and enzyme levels in various tissues, which had been reported by Zelko *et al*. (2002), and Limón-Pacheco and Gonsebatt (2009). As expected, SODs had the highest expressions in muscle, followed by brain, liver, kidney, gut, and heart, while the lowest in spleen and lung. Meanwhile, SOD activity was found to be higher in the brain, liver, muscle, kidney, gut and heart than in the other two tissues. Similar tissue-specific patterns were also clearly confirmed in some fish species. In *H. molitrix*, SOD1 is abundantly expressed in the liver and spleen, however SOD2 got the highest expression level in the gills followed by spleen and liver (Zhang *et al.*, 2011).

The two SODs had the highest levels of expression in *T. obscurus* liver (Kim *et al.*, 2010), as well as SOD2 highly expressed in the heart, followed by brain, gill, and muscle in *Hemibarbus mylodon* (Cho *et al.*, 2009). Furthermore, the eastern Pacific green turtle *Chelonia mydas agassizii* also showed the highest SOD activity in liver and muscle (Valdivia *et al.*, 2007). However, the enzyme activity for SODs in muscle and SOD1 in gut did not correspond to the mRNA expression level. Previous studies had indicated that the activity of SOD may be regulated during post-translational and transcriptional processes (Kim *et al.*, 2011). In general, the mRNA level and activity of SOD in aquatic organisms may be significantly affected by intracellular conditions, tissue type, species, and environmental oxidative cues (Zelko *et al.*, 2002; Lushchak, 2011b).

Because SODs constitute the first and most important line of antioxidant enzyme defense systems against ROS, the sequence and structure of SODs have been reported to be highly conserved among aerobic organisms (Zelko *et al.*, 2002). Presently, oxidative stress and SODs have been implicated in various pathological states involving inflammatory diseases, neurological disorders, ischemia/reperfusion, cancer and ageing (McCord and Edeas, 2005). $SOD1^{-/-}$ mice exhibited increased motor neuron loss after axonal injury compared to normal mice (Reaume *et al.*, 1996). Overexpression of human Mn-SOD in transgenic mice reduced neuronal death against ischemic injury (Keller *et al.*, 1998). Additionally, overproduction of SODs was associated with decreased oxidative damage and extended life span in yeast and fruit fly, highlighting the central role of SODs in life span regulation (Landis and Tower, 2005).

In conclusion, this work cloned the full-length cDNA sequences of SOD1 and SOD2 from *C. reevesii* for the first time. Both SOD genes contained well-conserved metal binding domains and signature sequences. Multiple alignments and phylogenetic analyses revealed that SODs proteins were conserved in structure during evolution. Both mRNA expression and enzyme activity measurements revealed that SODs were widely expressed in all tissues. The availability of sequences and tissue expression patterns would open a window for further studies on turtle's SODs at molecular level.

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